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The Transcription Factor FoxO1 Sustains Expression of the Inhibitory Receptor PD-1 and Survival of Antiviral CD8$^+$ T Cells during Chronic Infection

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SUMMARY

Protein kinase B (also known as AKT) and the mechanistic target of rapamycin (mTOR) are central regulators of T cell differentiation, proliferation, metabolism, and survival. Here, we show that during chronic murine lymphocytic choriomeningitis virus infection, activation of AKT and mTOR are impaired in antiviral cytotoxic T lymphocytes (CTLs), resulting in enhanced activity of the transcription factor FoxO1. Blockade of inhibitory receptor programmed cell death protein 1 (PD-1) in vivo increased mTOR activity in virus-specific CTLs, and its therapeutic effects were abrogated by the mTOR inhibitor rapamycin. FoxO1 functioned as a transcriptional activator of PD-1 that promoted the differentiation of terminally exhausted CTLs. Importantly, FoxO1-null CTLs failed to persist and control chronic viral infection. Collectively, this study shows that CTLs adapt to persistent infection through a positive feedback pathway (PD-1 → FoxO1 → PD-1) that functions to both desensitize virus-specific CTLs to antigen and support their survival during chronic viral infection.

INTRODUCTION

Chronic viral infection is a global health concern contributing to millions of deaths annually (Virgin et al., 2009). Viruses that cause chronic infection have evolved strategies to evade immune responses. The persistence of antigen can lead to alterations in cytotoxic T lymphocyte (CTL) proliferation, survival, effector functions, and gene expression that lead to the differentiation of dysfunctional or "exhausted" CTLs (Wherry, 2011). Exhausted CTLs that arise during certain chronic infections and cancers are characterized by impaired production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2), reduced cytotoxicity, and elevated surface expression of a number of inhibitory receptors, most notably programmed cell death protein 1 (PD-1) (Baitsch et al., 2011; Barber et al., 2006; Wherry, 2011). Upon interaction with its ligands PD-L1 or PD-L2, PD-1 can inhibit proximal T cell antigen receptor (TCR) signaling and suppress CTL function (Chemnitz et al., 2004; Keir et al., 2008; Mueller et al., 2010; Parry et al., 2005; Wei et al., 2013; Yokosuka et al., 2012; Zinselmeyer et al., 2013). Importantly, the induction of PD-1 and CTL exhaustion during chronic viral infection helps to balance the benefits of antiviral responses and viral control with the costs of immunopathology to the host (Barber et al., 2006; Frebel et al., 2012; Mueller et al., 2010; Zinselmeyer et al., 2013).

Evidence strongly points to a central role for sustained TCR signaling in fine-tuning the expression of PD-1 and many other genes that affect the function and homeostasis of virus-specific CTLs during chronic viral infection (Kao et al., 2011; Keir et al., 2008; Paley et al., 2012; Riley, 2009; Shin et al., 2007, 2009). However, it is unclear how TCR signaling is integrated with transcriptional changes that regulate these processes in CTLs during chronic infection. The activation of phosphoinositide 3-kinase (PI3K), protein kinase B (also known as AKT), and the mechanistic target of rapamycin (mTOR) either as part of mTOR complex 1, mTORC1, or mTORC2 by T cell, cytokine, and costimulatory receptors are of particular interest because they function in parallel pathways to control many aspects of T cell differentiation, proliferation, function, and survival (Finlay and Cantrell, 2011; Michalek and Rathmell, 2010; Pearce and Pearce, 2013; Powell and Delgoffe, 2010; Rao et al., 2010). Additionally, activation of PI3K, AKT, and mTOR signaling can induce a metabolic switch toward anabolic metabolism and aerobic glycolysis in activated CD8$^+$ T cells that is transcriptionally coordinated in part by c-*myc* and hypoxia inducible factor-1 (HIF-1) (Doedens et al., 2013; Finlay et al., 2012; Frauwirth et al., 2002; Jacobs et al., 2008; Macintyre et al., 2011; Wang et al., 2011). PI3K, AKT, and mTOR activation can also enhance T-bet transcription factor expression and the expression of several effector molecules including IFN-γ and granzyme B (Macintyre et al., 2011; Rao et al., 2010; Tomasoni et al., 2011).

Ligation of the inhibitory receptor PD-1 on the surface of activated CTLs results in enhanced expression and/or recruitment of SHP-1, SHP-2, or PTEN phosphatases that dampens proximal TCR signaling and activation of AKT (Patsoukis et al., 2013; Riley, 2009; Yokosuka et al., 2012; Zinselmeyer et al., 2013). Importantly, blockade of PD-1:PD-L1 interactions promotes the expansion of antiviral CTLs and improves viral control during viral infection (Barber et al., 2006). These findings have made PD-1 a

prime therapeutic target for enhancing T cell responses during certain forms of chronic infection and cancer (Speiser et al., 2014). However, the pertinent signaling pathways that underlie the recovery of T cell responses by PD-1:PD-L1 blockade in vivo are not known; such information could offer important insights into the etiology of CTL exhaustion and might also provide a basis for the development of therapies for treating chronic diseases.

Although signaling through PI3K, AKT, and mTOR enhances effector CTL differentiation, it suppresses the differentiation and maturation of memory CTLs (Araki et al., 2009; Hand et al., 2010; Kim et al., 2012; Pearce et al., 2009; Rao et al., 2010). This occurs, in part, because AKT phosphorylation inhibits the nuclear activity of FoxO transcription factors, namely FoxO1, which positively regulates several genes involved in naive and memory T cell survival and trafficking including *Il7ra*, *Ccr7*, *Klf2*, *Sell* (CD62L), *Tcf7*, *Eomes*, and *Bcl2* (Kerdiles et al., 2009, 2010; Kim et al., 2012, 2013; Hess Michelini et al., 2013; Ouyang et al., 2009, 2010, 2012; Rao et al., 2012; Sullivan et al., 2012a, 2012b; Tejera et al., 2013). Moreover, FoxO1 counterbalances effector CTL differentiation via repression of T-bet, IFN-γ, and granzyme B expression (Hess Michelini et al., 2013; Ouyang et al., 2012; Rao et al., 2012). However, the precise roles of FoxO1, AKT, and mTOR signaling in controlling functional exhaustion, metabolism, and differentiation of $CD8^+$ T cells during chronic infection has not been explored.

In this study, we identified critical roles for PI3K, AKT, and mTOR signaling and FoxO1 transcriptional activity in the homeostasis and differentiation of $CD8^+$ T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection. We found that sustained antigenic signaling from persisting virus reduced, rather than enhanced, TCR signal transduction and activation of AKT and mTOR. This suppression of mTOR activation was largely due to PD-1:PD-L1 signaling, and rapamycin abolished the restoration of CTL responses and overall therapeutic efficacy of α-PD-L1 blockade. Consequently, suppression of AKT led to increased FoxO1 nuclear activity, which was necessary to sustain PD-1 expression and acquisition of terminally exhausted states during chronic infection. However, FoxO1 was also required for Bcl2 expression and the survival of CTLs during chronic infection. These results suggested that increased FoxO1 activity and expression of PD-1 are important adaptations by virus-specific CTLs during persistent infection that support their homeostasis.

RESULTS

TCR Activation of PI3K, AKT, and mTOR and Markers of Anabolic Metabolism Are Poorly Sustained in Exhausted $CD8^+$ T Cells

To better understand the regulation of PI3K, AKT, and mTOR signaling in antigen-specific CTLs during acute (LCMV-Arm) and chronic (LCMV-Cl13) viral infection, we combined adoptive T cell transfer techniques and phospho-flow cytometry (Figure 1A). Although peak phosphorylation of S6, a readout of mTOR activity (present at day 5 postinfection [p.i.]), was reduced in CTLs during LCMV-Cl13 compared to LCMV-Arm infection, p-S6 was not sustained in CTLs during LCMV-Cl13 infection despite the persistence of virus (Figure 1B; Sullivan et al., 2012b). These data led us to hypothesize that CTLs during chronic infection might be impaired in their ability to sustain the phosphorylation of PI3K, AKT, and mTOR, and that this might contribute to their functional exhaustion.

To address this possibility, we directly compared the TCR signaling capacity between two populations of gp33-41-specific TCR transgenic (P14) CTLs isolated from LCMV-Arm or LCMV-Cl13 infection. By 8 days p.i., CTLs from LCMV-Cl13 infection demonstrated a loss in TCR responsiveness compared to those from LCMV-Arm infection as measured by reduced phosphorylation of both proximal (e.g., $Zap70^{319}$) and distal signaling molecules, such as $ERK^{202/204}$, AKT^{308} and AKT^{473}, $m\text{-}TOR^{2448}$, and their downstream targets $S6^{235/236}$ and $FoxO1^{24}$ and $Foxo3a^{32}$ (referred to as FoxO1/3a) (Figures 1C and 1D, Figures S1A–S1C available online, includes specificity controls for p-S6 and p-FoxO1/3a by PI3K, AKT, and mTOR). Importantly, the defect in TCR signaling was sustained at later time points (d21 p.i.) and could not be solely explained by decreased expression of the TCR itself or rescued by CD28 costimulation (data not shown). However, phorbol ester or phosphatase inhibitor treatment of day 8 CTLs from LCMV-Cl13 infection could at least partially restore phosphorylation of p-Zap70, p-S6, and p-ERK, suggesting active inhibition of TCR signaling (data not shown). Taken together, our data point to a proximal signaling defect in exhausted CTLs that results in dampened phosphorylation of S6, AKT, and FoxO1/3a (Powell et al., 2012).

Next, to determine whether the reduction in PI3K, AKT, and mTOR kinase activity in CTLs during chronic infection was a general feature associated with CTL exhaustion, we examined the ability of CTLs from acute and chronic viral infections to respond to cytokine stimulation. Although we found that the ability of CTLs from LCMV-Cl13-infected mice to phosphorylate STAT5 and STAT3 transcription factors downstream of IL-2, IL-15, and IL-21 was not significantly impaired relative to CTLs from LCMV-Arm-infected mice, their ability to activate the AKT and mTOR was (Figures S1D–S1F). Taken together, these results demonstrate that the progressive loss of effector functions that occurs in CTLs during chronic LCMV infection (Wherry, 2011) correlates with their impaired ability to activate the PI3K, AKT, and mTOR signaling pathways.

Chronic antigen stimulation contributes to many of the phenotypic and molecular changes that define exhausted CTLs during chronic infection (Angelosanto et al., 2012; Brooks et al., 2006; Kao et al., 2011; Paley et al., 2012; Shin et al., 2007). Therefore, to directly test whether persistent antigen contributed to TCR desensitization in virus-specific $CD8^+$ T cells during chronic viral infection, we varied the duration of antigen exposure by transferring functional P14 CTLs from acute LCMV-Arm-infected animals into recipients infected 8 days previously with either (1) LCMV-Arm, (2) LCMV-Cl13, or (3) LCMV-Cl13-V35A, a mutant strain of LCMV-Cl13 that contains an amino acid substitution in the gp33 epitope and is not recognized by the P14 TCR (Puglielli et al., 2001). Importantly, infection with LCMV-Cl13-V35A resulted in comparable viremia and elevated PD-1 expression on gp276- and 396-specific CTLs (data not shown; Shin et al., 2007). Indeed, TCR-dependent phosphorylation of S6, FoxO1/3a, and ERK was elevated in P14 CTLs engrafted into

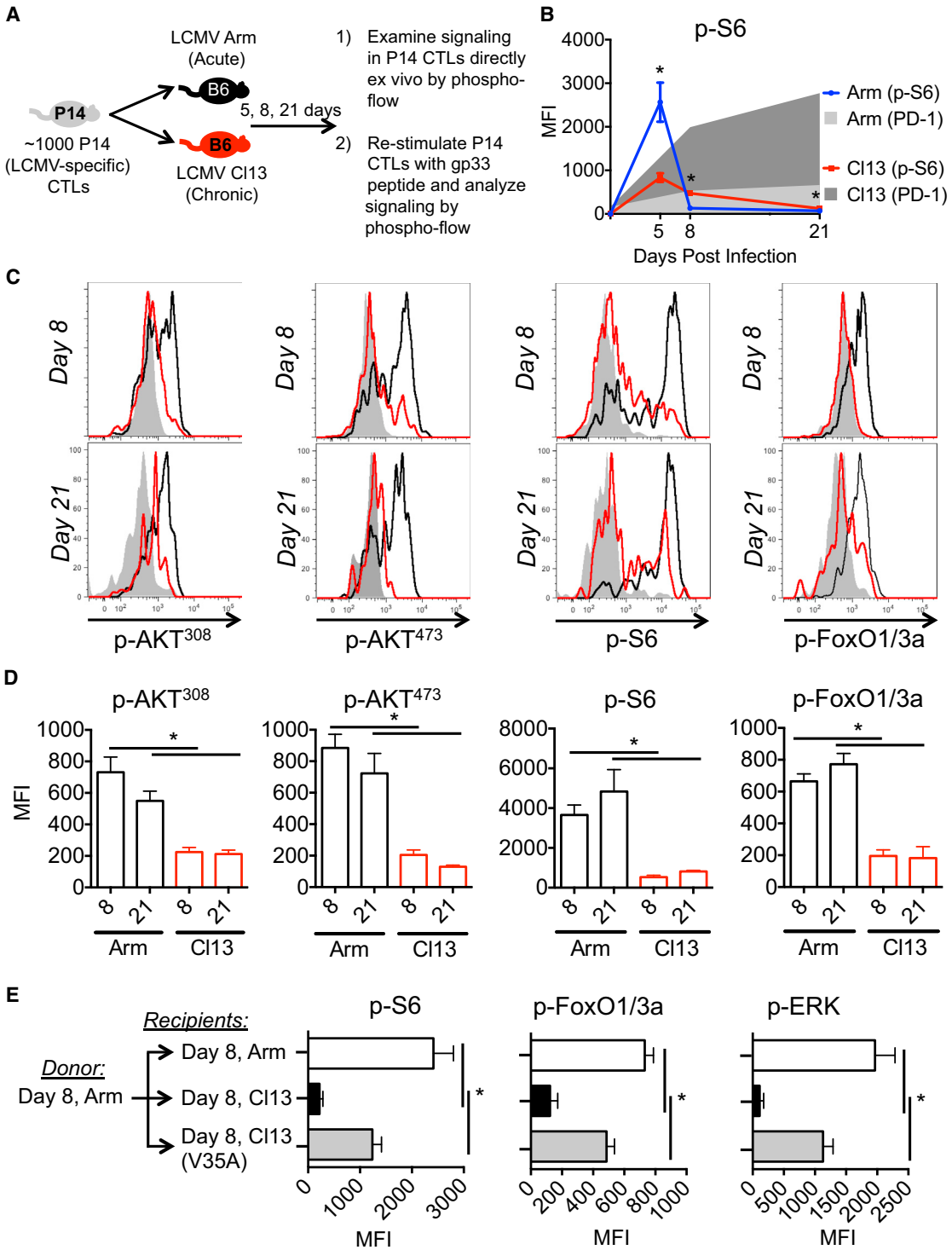

Figure 1. Persistent Antigen Suppresses TCR Activation of AKT and mTOR Signaling in CTLs during Chronic Infection

(A) Experimental approach used to study TCR and cytokine signaling in LCMV-specific P14 CTLs during LCMV Armstrong (acute) and LCMV-Clone 13 (chronic) infection by phospho-flow.

(B) p-S6 (line) and PD-1 (shaded) expression in P14 CTLs directly ex vivo 5, 8, and 21 days after LCMV-Arm (blue, light gray shading) or LCMV-Cl13 (red, dark gray shading) infection.

(C) Representative histograms of P14 CTLs from day 8 or day 21 after LCMV-Arm (black line) or LCMV-Cl13 (red line) infection were stimulated with gp33 peptide for 60 min and p-AKT^{308}, p-AKT^{473}, p-S6, and p-FoxO1/3a were measured by phospho-flow. Shaded histograms are unstimulated CTLs from LCMV-Cl13 infection.

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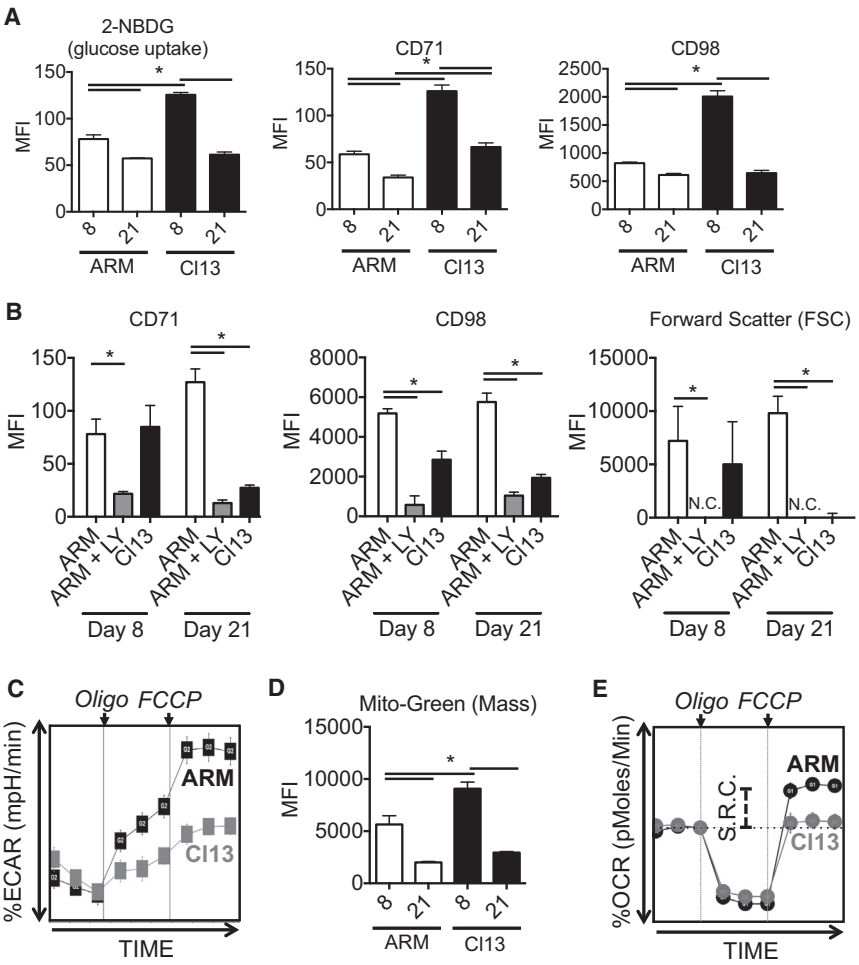

Figure 2. Features of Anabolic Metabolism Are Poorly Sustained in Exhausted CTLs during Chronic Infection

(A) 2-NDBG staining and CD71 and CD98 expression in P14 CTLs at day 8 or 21 after LCMV-Arm or LCMV-Cl13 infection were measured directly ex vivo by flow cytometry.

(B) P14 CTLs at day 8 or 21 after from LCMV-Arm or LCMV-Cl13 infection were stimulated with gp33 peptide ±25 nM LY294002 (PI3K inhibitor) for 16–24 hr, and CD71 and CD98 expression and forward scatter (FSC) was measured by flow cytometry.

(C) Seahorse extracellular flux analysis showing the ECAR of purified P14 CTLs at day 8 after LCMV-Arm or LCMV-Cl13 infection (normalized to baseline) after the addition of oligomycin (ATPase inhibitor) and FCCP (mitochondrial uncoupling agent).

(D) Mitochondrial green (mass) staining in P14 CTLs via flow cytometry as in (A).

(E) Seahorse extracellular flux analysis showing the OCR of purified P14 CTLs at day 8 after LCMV-Arm or LCMV-Cl13 infection (normalized to baseline) after the addition of oligomycin (ATPase inhibitor) and FCCP (mitochondrial uncoupling agent). SRC is indicated as the difference between baseline OCR and after the addition of FCCP.

Data are representative of three independent experiments that included three to five mice/group. Error bars are mean ± SEM. See also Figure S2.

LCMV-Arm- or LCMV-Cl13-V35A- as compared to LCMV-Cl13-infected mice (Figure 1E). These findings demonstrate that prolonged contact with antigen underlies the loss in TCR responsiveness and downstream signaling in CTLs during chronic infection.

Consistent with their inability to sustain robust mTOR activity (Figure 1B), at day 21 p.i., virus-specific CTLs from chronic LCMV-Cl13 infection were unable to maintain properties of anabolic metabolism such as robust glucose uptake (2-NBDG) and expression of CD71 (transferrin receptor) and CD98 (amino acid transporter) (Figure 2A; Finlay and Cantrell, 2011; Finlay et al., 2009). Furthermore, although CTLs from acute infection were able to upregulate CD71 and CD98 and initiate blastogenesis in response to TCR restimulation in a PI3K-, AKT-, and mTOR-dependent manner, CTLs from LCMV-Cl13 failed to do so (Figures 2B and S2A). Although glucose uptake was somewhat higher at day 8 in CTLs from chronic relative to acute infection, their baseline extracellular acidification rates (ECAR, a measure of glycolysis) were similar (Figure S2B). Additionally, CTLs from chronic infection were less efficient in their ability to engage glycolysis when mitochondrial ATP synthesis was blocked by oligomycin (Figure 2C; van der Windt et al., 2013). Similarly, at day 8 p.i., despite an increase in mitochondrial mass in CTLs from chronic infection (Figure 2D), their baseline oxygen consumption rates (OCR, a measure of mitochondrial oxidative phosphorylation) were comparable to CTLs from acute infection (Figure S2B). Moreover, when CTLs from chronic infection were treated with the mitochondrial uncoupling reagent FCCP, their maximal mitochondrial respiration (i.e., spare respiratory capacity [SRC]) was reduced compared to those from acute infection (Figure 2E; van der Windt et al., 2013). Collectively, these data suggest that exhausted CTLs display deficiencies in both glycolytic and oxidative metabolism, which further underscore the decline in TCR-dependent PI3K signaling in virus-specific CTLs during chronic LCMV-Cl13 infection.

Therapeutic PD-L1 Blockade Requires mTOR Activation

PD-1 is markedly upregulated on exhausted CTLs and can inhibit proximal TCR signaling and distal AKT, mTOR, S6, and ERK phosphorylation (Francisco et al., 2009; Parry et al., 2005; Sheppard et al., 2004; Yokosuka et al., 2012). Moreover, blocking

(D) Cumulative bar graphs showing the mean fluorescence intensity (MFI) of phospho-flow data from (C).

(E) P14 CTLs from day 8 LCMV-Arm infection were transferred into infection-matched LCMV-Arm (white), LCMV-Cl13 (black), or LCMV-Cl13-V35A (gray) recipients. At day 15 (7 days after transfer), P14+ CTLs were stimulated with gp33 peptide for 60 min and p-S6, p-FoxO1/3a, and p-ERK were measured by phospho-flow.

Data are representative of three independent experiments that included three to five mice/group. Error bars are mean ± SEM. See also Figure S1.

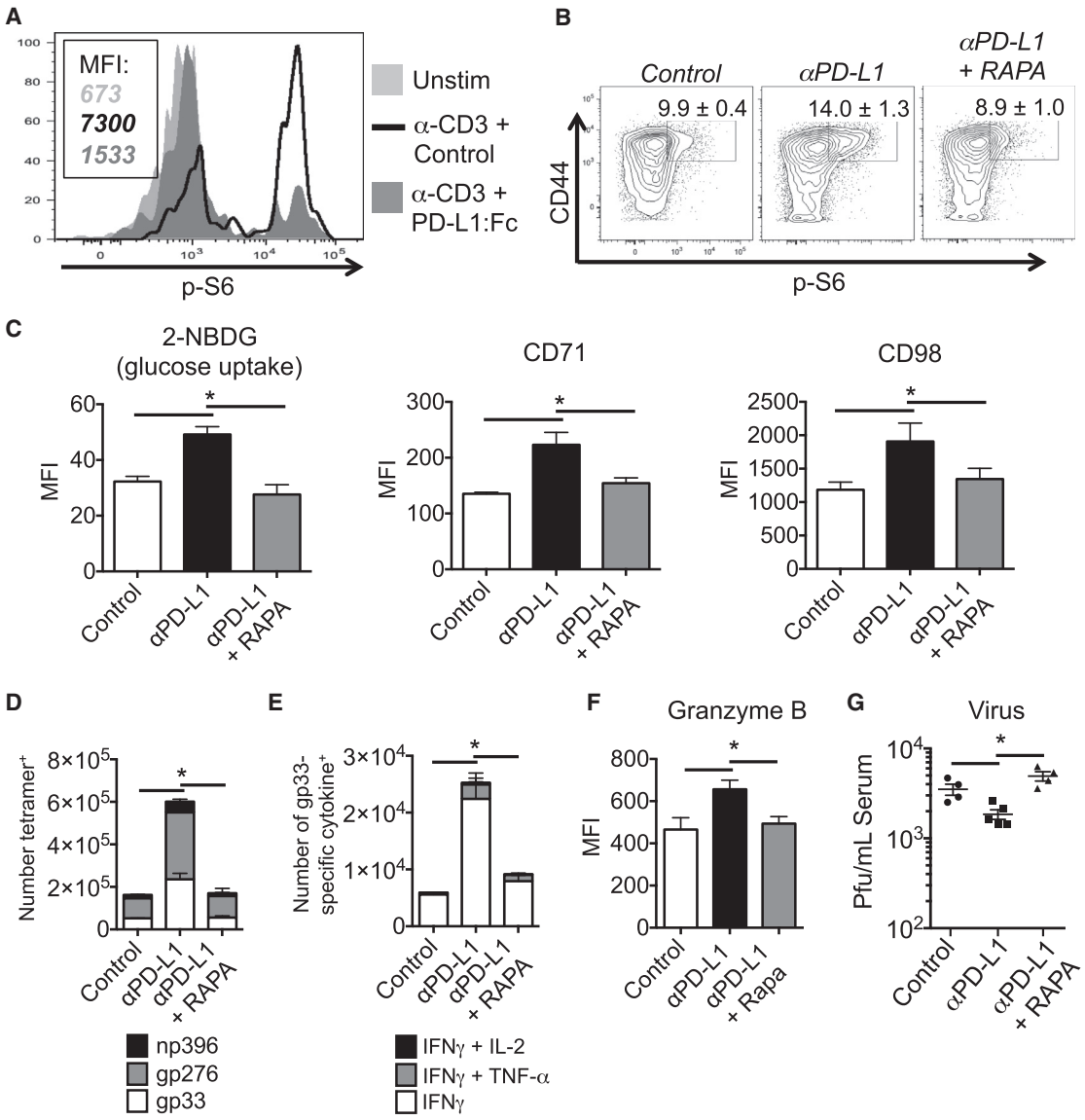

Figure 3. PD-1 Suppresses mTOR Signaling and Markers Associated with Anabolic Metabolism in Exhausted CTLs during Chronic Infection

(A) Histograms show amounts of phosphorylated S6 (from day 8 after LCMV-Arm infection) 60 min after anti-CD3 (3 μg/ml) + PD-L1:Fc (dark gray shading), CD3 (3 μg/ml) + control IgG (20 μg/ml) (black line), or unstimulated (light gray shading). Shaded histograms are unstimulated CTLs. MFIs are indicated.

(B–F) LCMV-Cl13-infected mice were PBS treated (white shading) or treated at day 28 p.i. with α-PD-L1 blocking mAb (200 μg/mouse) (black shading) alone or in combination with rapamycin (100 μg/kg) (gray shading) for 7 days.

(B and C) Mice were then analyzed for (B) the percentage of p-S6^{+}CD8^{+}CD44^{hi} T cells and (C) intracellular staining via 2-NDBG, and surface expression of CD71 and CD98 in gp33 tetramer^{+} CTLs.

(D and E) Stacked bar graphs showing the number of tetramer-positive CTLs (gp33, gp276, and np396) (D), and the number of gp33-specific cytokine-producing CTLs (E).

(F) The expression of granzyme B in gp33-specific CTLs as in (B).

(G) Viral titers in the serum as determined by plaque assay.

Data are representative of three independent experiments that included three to five mice/group. Error bars are mean ± SEM.

PD-1:PD-L1 interactions can restore TCR signaling (Fife et al., 2009; Zinselmeyer et al., 2013) and boost antiviral T cell responses and viral control during LCMV-Cl13 infection (Barber et al., 2006). First, we confirmed the ability of PD-1 to suppress mTORC1 activity (based on p-S6 staining) in virus-specific CTLs after TCR stimulation (Figure 3A). Next, to examine whether PD-1 suppression of mTOR activity was relevant in antiviral CTLs in vivo, LCMV-Cl13-infected mice were treated with a blocking α-PD-L1 mAb, either with or without the mTORC inhibitor rapamycin. Anti-PD-L1 mAb treatment augmented the amounts of p-S6$^{235/236}$, CD98, CD71, and glucose uptake in CTLs (Figures 3B and 3C; Finlay et al., 2009). The increase in mTOR activity was accompanied by a marked increase in the frequency and number of IFN-γ- and granzyme B

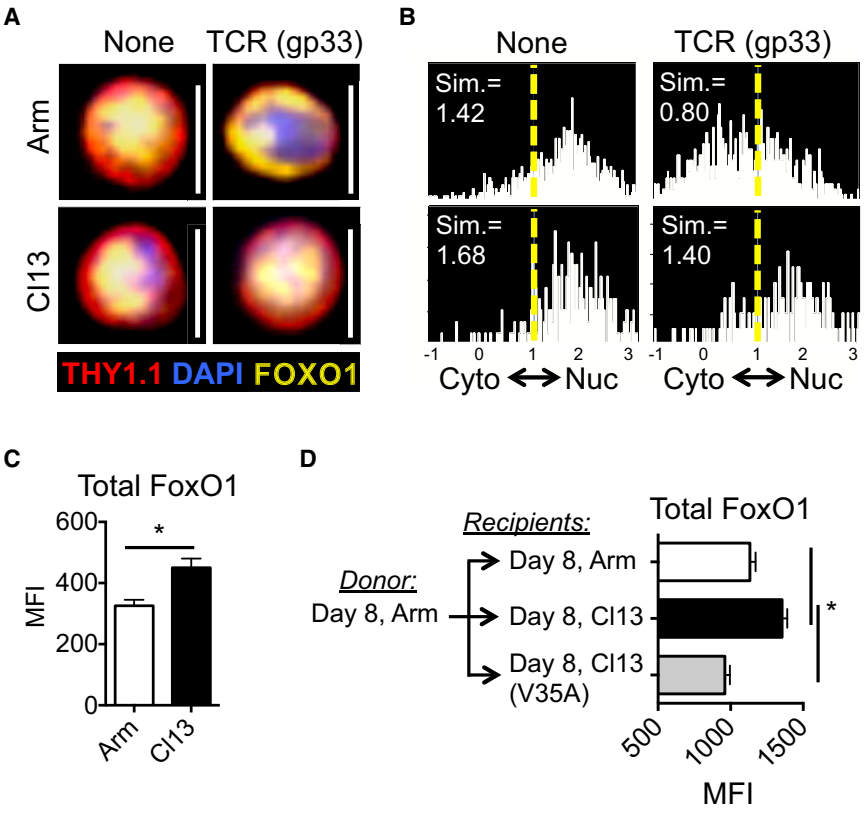

Figure 4. Chronic Antigen Promotes the Expression and Nuclear Retention of FoxO1 in CD8+ T Cells during Chronic Infection

(A) Representative image of P14 CTLs from day 8 after LCMV-Arm or LCMV-Cl13 infection were stimulated with gp33 peptide for 60 min and nuclear versus cytoplasmic FoxO1 was determined by the Amnis ImagestreamX (Amnis). Scale bars represent 10 μm.

(B) Histograms showing the cumulative similarity score (Sim) of FoxO1 and DAPI staining to measure nuclear localization as in (A).

(C) Total FoxO1 expression in gp33-specific CTLs was analyzed by intracellular staining at day 21 after LCMV-Arm and LCMV-Cl13 infection.

(D) P14 CTLs from day 8 LCMV-Arm infection were transferred into infection-matched LCMV-Arm, LCMV-Cl13, or LCMV-Cl13-V35A recipients. At day 15 (7 days after transfer), P14+ CTLs were analyzed for total FoxO1 expression by intracellular staining.

Data are representative of three independent experiments that included three to five mice/group. Error bars are mean ± SEM.

(GzmB)-producing virus-specific CTLs and decrease in viral load (Figures 3D–3G). Importantly, rapamycin abrogated the beneficial effects of α-PD-L1 blockade on viral control and virus-specific T cell responses, including the aforementioned markers of anabolic metabolism (Figures 3B–3F). Taken together, these data demonstrate that PD-1 suppression of the mTOR pathway contributes to CTL exhaustion in vivo and that recovery of mTOR activity is a part of the therapeutic effects of α-PD-L1 during chronic LCMV-Cl13 infection.

Expression and Nuclear Retention of the Transcription Factor FoxO1 Is Enhanced in Exhausted CD8+ T Cells

FoxO1 supports the differentiation of regulatory T cells, suppresses effector functions, and promotes the differentiation and survival of long-lived memory CD8+ T cells (Hedrick et al., 2012; Kim et al., 2013; Hess Michelini et al., 2013; Ouyang et al., 2012; Tejera et al., 2013). FoxO transcription factors are phosphorylated and inhibited by AKT in response to mTORC2 activation (Biggs et al., 1999; Brunet et al., 1999; Calnan and Brunet, 2008; Rao et al., 2012). Therefore, we questioned whether the suppression of AKT signaling in CTLs during chronic viral infection results in enhanced FoxO1 activity. Consistent with decreased p-FoxO1/3a after TCR stimulation (Figures 1 and S1C), CTLs from LCMV-Cl13 infection displayed enhanced nuclear retention of FoxO1 after TCR stimulation (Figures 4A and 4B) and contained elevated amounts of FoxO1 protein (Figure 4C) compared to those from acute LCMV-Arm infection. Transfer of P14 cells from LCMV-Arm-infected animals into recipients infected 8 days previously with LCMV-Arm, -Cl13, or -Cl13-V35A confirmed that FoxO1 expression was upregulated in an antigen-dependent manner during chronic viral infection (Figure 4D). Furthermore, PD-1 ligation on the surface of effector CTLs (as in Figure 3A) could also effectively suppress the phosphorylation of FoxO1/3a in response to TCR stimulation (data not shown). These data suggest that persistent antigenic stimulation and PD-1 signaling dampens TCR-dependent activation of AKT and mTORC2, leading to enhanced nuclear accumulation of FoxO1 in antiviral CTLs during chronic infection.

FoxO1 Regulates the Homeostasis of Virus-Specific CD8+ T Cells during Chronic Viral Infection

To determine whether FoxO1 played a role in CTL exhaustion during chronic infection, we conditionally deleted *Foxo1* from virus-specific CD8+ T cells during LCMV-Cl13 infection by using *Foxo1*$^{fl/fl}$*Gzmb-cre*$^{+}$ mice (referred to as *FoxO1*$^{fl/fl}$). Deletion of *FoxO1* impaired virus-specific CTL responses such that their frequency and number were reduced by nearly 50% at day 21 p.i. compared to wild-type (WT) *Foxo1*$^{+/+}$*Gzmb*-cre^{+} littermate controls (referred to as *FoxO1*$^{+/+}$) (Figures 5A, 5B, and S3A). Cytokine production by *FoxO1*$^{fl/fl}$ CTLs was only modestly affected, but the expression of granzyme B, a FoxO1-repressed target gene (Macintyre et al., 2011; Hess Michelini et al., 2013; Rao et al., 2012; Tejera et al., 2013), was markedly increased (Figures 5C, 5D, and S3B). Despite this, viral loads remained significantly higher in *FoxO1*$^{fl/fl}$ relative to *FoxO1*$^{+/+}$ mice, probably owing to a combination of decreased proliferation and survival of LCMV-specific CTLs in the absence of FoxO1 as suggested by Ki67 and by the ratio of Bim to Bcl-2 staining, respectively (Figures 5E–5G and S3C; Kim et al., 2013; Tejera et al., 2013). Taken together, these data propose an intriguing model wherein the suppression of mTOR and AKT, and resulting increase in

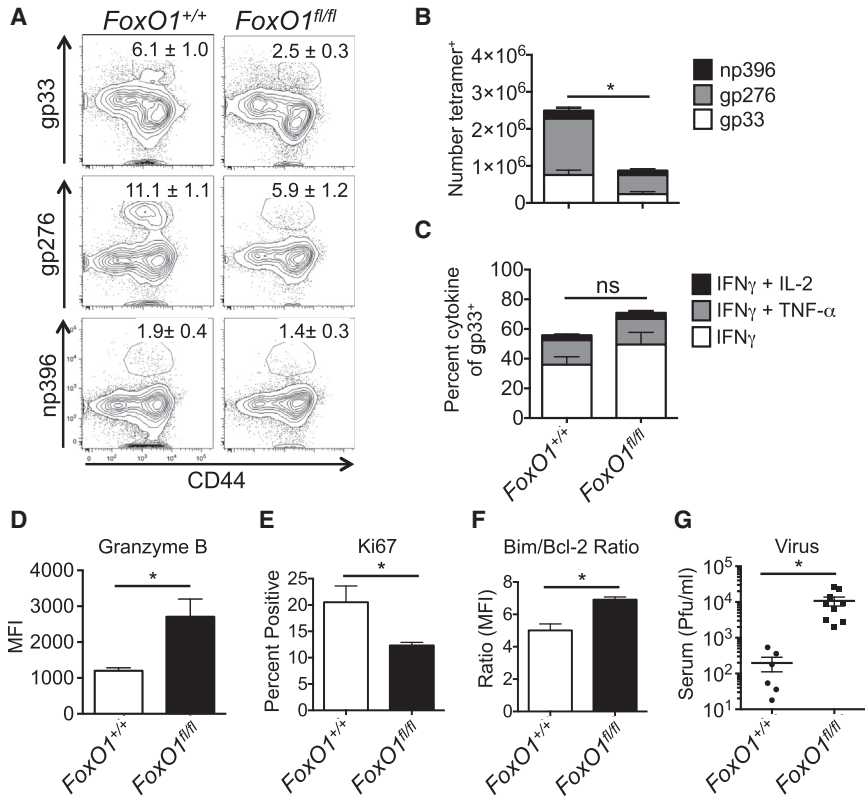

Figure 5. FoxO1 Sustains Virus-Specific CD8+ T Cell Responses during Chronic Viral Infection

(A and B) *Foxo1*$^{+/+}$*Gzmb-cre*$^{+}$ (*FoxO1*$^{+/+}$) and *Foxo1*$^{fl/fl}$*Gzmb-cre*$^{+}$ (*FoxO1*$^{fl/fl}$) mice were infected with LCMV-Cl13 and at day 21 p.i., the frequency (A) and number (B) of tetramer^{+} CTLs (gp33, gp276, and np396) was determined in the spleen by flow cytometry.

(C) *FoxO1*$^{+/+}$ and *FoxO1*$^{fl/fl}$ CTLs as in (D) were restimulated with gp33 peptide in the presence of brefeldin A and cytokine production was measured by flow cytometry.

(D–G) *FoxO1*$^{+/+}$ and *FoxO1*$^{fl/fl}$ gp33-tetramer^{+} CTLs as in (A) were examined for expression of (D) granzyme B, (E) Ki67, and (F) Bim:Bcl-2 ratio by flow cytometry, and (G) serum viral titers were determined by plaque assay.

Data are cumulative from four independent experiments (*FoxO1*$^{+/+}$, n = 10–13; *FoxO1*$^{fl/fl}$, n = 10–17). Error bars are mean ± SEM. See also Figure S3.

FoxO1 activity, dampens effector molecule expression but simultaneously promotes virus-specific CTL survival during chronic infection.

FoxO1 Is Necessary for the Differentiation of PD-1^{hi}Eomes^{hi} Terminally Exhausted CTLs

During chronic LCMV infection, the CTL pool is maintained by periodic proliferation and conversion of PD-1^{int}T-bet^{hi} → PD-1^{hi}Eomes^{hi} cells (Kao et al., 2011; Paley et al., 2012). Together these subsets cooperate to maintain a durable and partially effective CD8^{+} T cell response during chronic infection (Paley et al., 2012). Upon further inspection, we found that FoxO1 expression was increased in PD-1^{hi} CTLs that also expressed higher amounts of Eomes, but lower amounts of T-bet (Figures 6A and 6B; Paley et al., 2012). We posited that FoxO1 might regulate the progression and balance between PD-1^{int}T-bet^{hi} and PD-1^{hi}Eomes^{hi}CD8^{+} T cell subsets during chronic infection because it can promote Eomes and suppress T-bet expression in acutely stimulated CD8^{+} T cells (Hess Michelini et al., 2013; Rao et al., 2010, 2012). Consistent with this model, at day 21 p.i., the expression of PD-1 and Eomes and frequency of PD-1^{hi} Eomes^{hi} CTLs were significantly reduced in the absence of FoxO1, while the proportion of PD-1^{lo} CTLs that expressed T-bet were markedly increased (Figures 6C–6E, data not shown). Adoptive transfer of *FoxO1*$^{fl/fl}$ P14 CTLs into wild-type mice infected with LCMV-Cl13 validated the cell-autonomous role for FoxO1 in regulating the formation of PD-1^{hi}Eomes^{hi} CTLs (Figure S4A). The phenotypes of the *FoxO1*$^{fl/fl}$ CTLs were more modest at day 8 p.i. compared to day 21 p.i.; for example, Eomes was lower than that of the *FoxO1*$^{+/+}$ CTLs, but no significant differences in PD-1 and T-bet expression were observed (Figure S4B). These data suggest that the dependency on FoxO1 for elevating Eomes, Bcl-2, and PD-1 and for repressing T-bet expression increases as chronic infection progresses. Probably, the improper regulation of these genes in *FoxO1*$^{fl/fl}$ cells impairs CTL maintenance over time (Figures 6F–6H; Kao et al., 2011; Paley et al., 2012; Rao et al., 2012).

FoxO1 Binds to and Promotes the Expression of PD-1 in CD8^{+} T Cells

Based on the decreased PD-1 expression in *FoxO1*$^{fl/fl}$CD8^{+} T cells, we hypothesized that, in addition to Eomes (Rao et al., 2012), FoxO1 might also directly control PD-1 (*Pdcd1* gene) transcription as CTLs transition from PD-1^{int} to PD-1^{hi} states during chronic LCMV infection. Examination of this possibility revealed that the upstream regulatory region of *Pdcd1*, named the PD-1 "C-region" that is trans-activated by the transcription factor NFATc1 (Oestreich et al., 2008), also contains a putative FoxO1 binding site (Figure 7A; Ouyang et al., 2012). The C-region also contains a candidate T-bet binding site and neighboring Blimp-1 binding site, and overexpression of T-bet or Blimp-1 can suppress PD-1 expression (Kao et al., 2011; Lu et al., 2014). By chromatin immunoprecipitation (ChIP), we observed FoxO1 binding to the C-region, but not the neighboring B-region in *Pdcd1* in in vitro activated CD8^{+} T cells (Figure 7B). Furthermore, overexpression of FoxO1 was sufficient to enhance the activity of a *Pdcd1* promoter luciferase reporter in Jurkat cells when both B- and C-regions were intact, but not when only the B-region was present (Figure 7C). In vitro, PD-1 expression in CD8^{+} T cells could be enhanced via treatments that increase FoxO1 activity such as inhibition of PI3K, AKT, or mTOR, or through inhibition of glycolysis with 2-deoxy-D-glucose (2-DG) (data not shown; Chang et al., 2013). To test the role of FoxO1 more directly, we overexpressed an

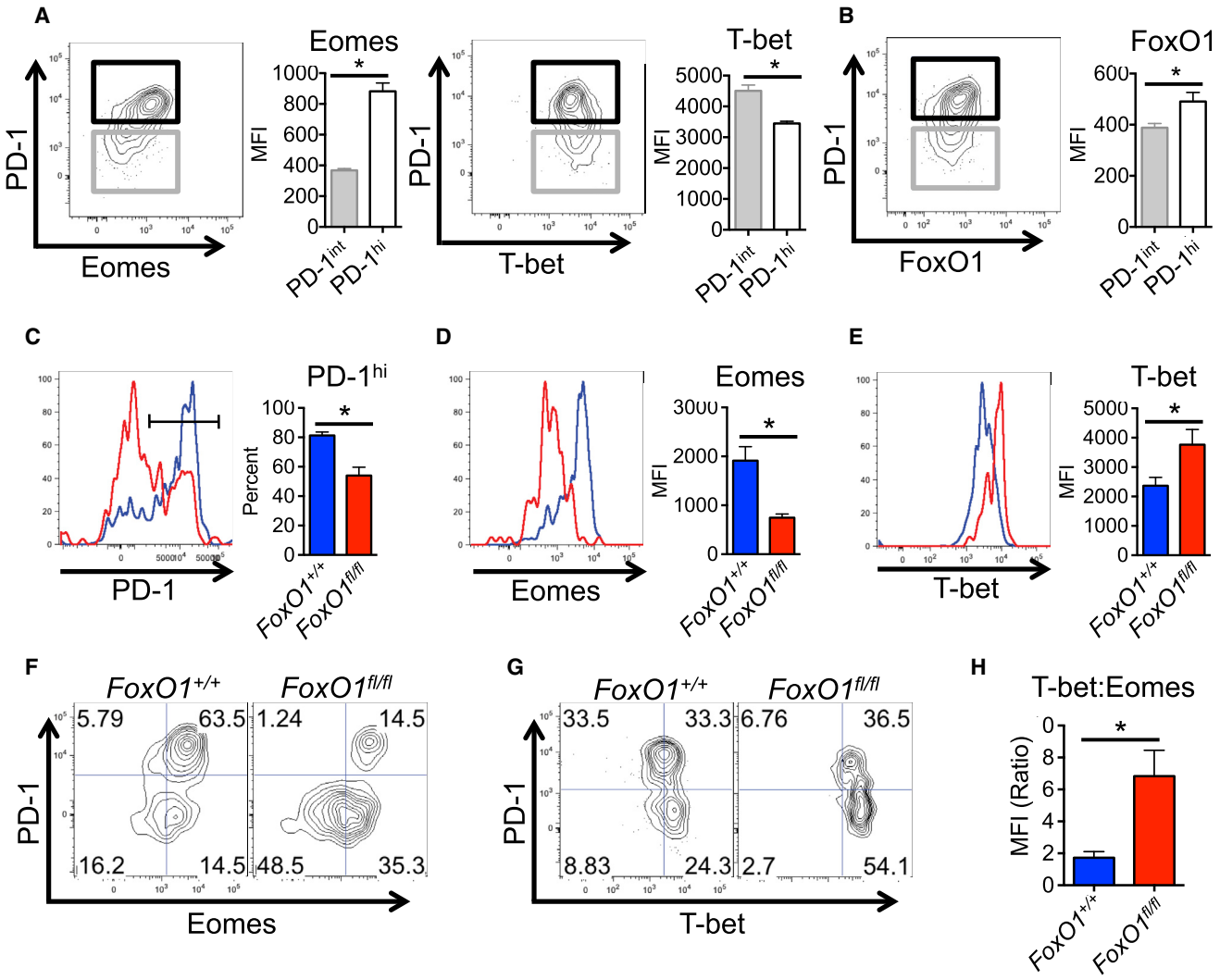

Figure 6. FoxO1 Regulates the Differentiation of PD-1^{hi}Eomes^{hi} and PD-1^{lo}T-bet^{hi} Populations

(A and B) Representative dot plots and cumulative bar graphs showing Eomes and T-bet (A) and FoxO1 (B) expression in PD-1^{hi} versus PD-1^{int} P14 CTLs at day 21 after LCMV-Cl13 infection as determined by flow cytometry (n = 7 mice/group).

(C–E) Representative histogram overlays and cumulative bar graphs showing (C) the frequency of PD-1^{hi} CTLs and the MFI of (D) Eomes and (E) T-bet in *FoxO1*$^{+/+}$ (blue) and *FoxO1*$^{fl/fl}$ (red) gp33 tetramer^{+} CTLs at day 21 after LCMV-Cl13 infection as determined by flow cytometry.

(F and G) Representative dot plots of *FoxO1*$^{+/+}$ and *FoxO1*$^{fl/fl}$ gp33-tetramer^{+} CTLs showing (F) PD-1 versus Eomes or (G) PD-1 versus T-bet expression at day 21 after LCMV-Cl13 infection.

(H) The ratio of T-bet to Eomes expression in *FoxO1*$^{+/+}$ and *FoxO1*$^{fl/fl}$ gp33-tetramer^{+} CTLs at day 21 after LCMV-Cl13 infection is shown.

Data are cumulative from four independent experiments (*FoxO1*$^{+/+}$, n = 10–13; *FoxO1*$^{fl/fl}$, n = 10–17). Error bars are mean ± SEM. See also Figure S4.

AKT-mediated phosphorylation-resistant mutant of FoxO1, FoxO1^{AAA}, in CTLs in vitro or in vivo during chronic LCMV-Cl13 infection. In vitro, constitutively active FoxO1^{AAA} was able to upregulate PD-1 expression, an effect that was enhanced further by antigenic restimulation (data not shown). In vivo, FoxO1^{AAA} was sufficient to augment the expression of PD-1, Eomes, and Bcl-2 while suppressing that of T-bet, effectively shifting the balance toward the differentiation of PD-1^{hi}Eomes^{hi} CTLs (Figures 7D and 7E, data not shown; Paley et al., 2012). Thus, FoxO1 appears to directly trans-activate the *Pdcd1* locus and promote the formation of more terminally exhausted PD-1^{hi}Eomes^{hi}CD8^{+} T cells during chronic viral infection.

DISCUSSION

This study identifies the relevance of fine-tuning of the PI3K, AKT, and mTOR signaling pathways in regulating the differentiation and function of virus-specific CD8^{+} T cells during chronic viral infection. Our work has characterized the decline in TCR signaling in exhausted CTLs that results in reduced mTORC1 and mTORC2 activity and inability to sustain high rates of aerobic glycolysis and anabolic metabolism. Reactivation of mTOR was necessary to bolster antiviral CTL proliferation and effector functions during PD-L1 blockade therapy, thereby placing mTOR on a central axis controlling CTL exhaustion. Importantly,

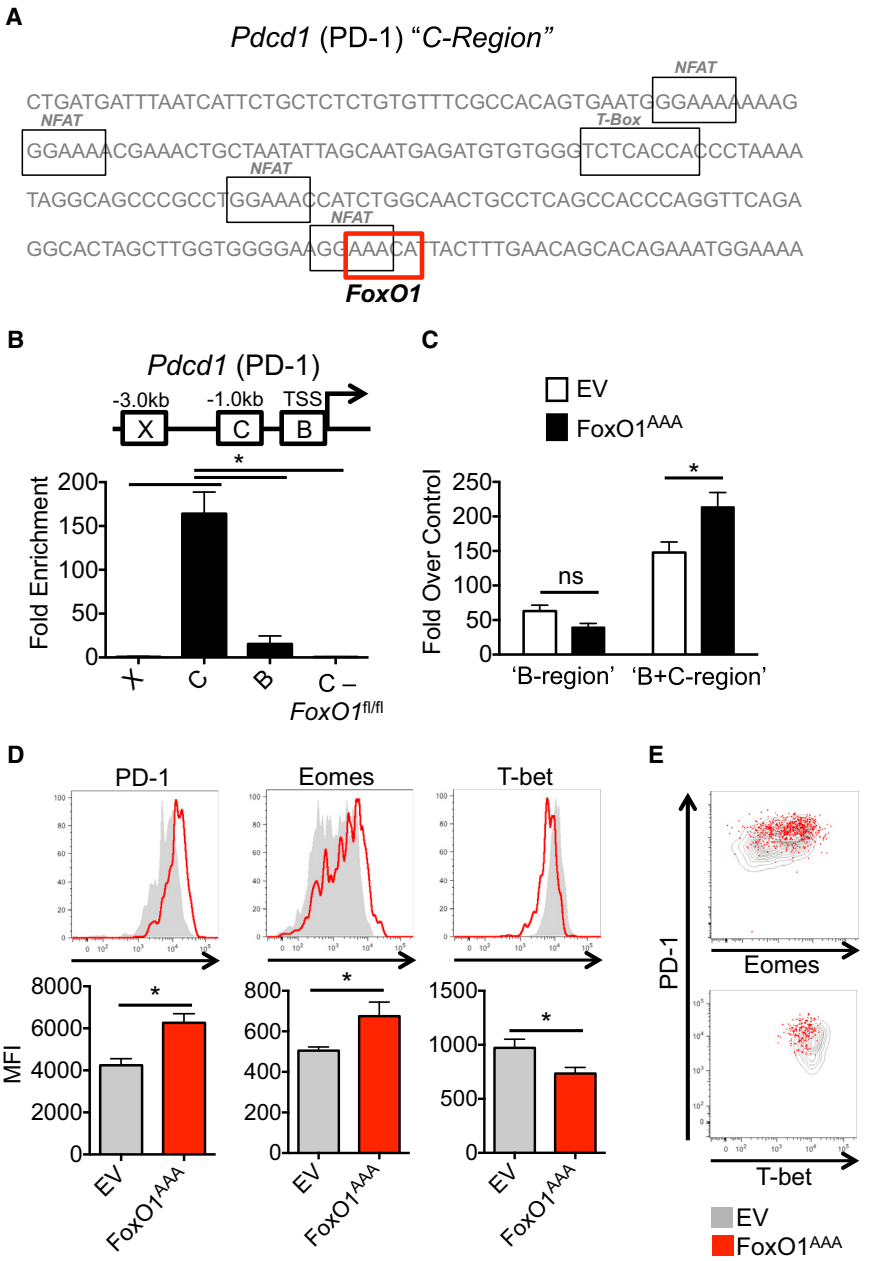

Figure 7. FoxO1 Directly Regulates PD-1 Expression in CD8+ T Cells and Promotes the Differentiation of PD-1hiEomeshiCD8+ T Cells during Chronic Infection

(A) Outline of putative NFAT, FoxO1, and T-box consensus binding motifs in the C-region of the *Pdcd1* locus (Oestreich et al., 2008).

(B) Chromatin immunoprecipitation (ChIP) analysis of FoxO1 binding to the B- and C-regions (Oestreich et al., 2008) of the *Pdcd1* promoter in day 3 in vitro activated P14 CTLs from *FoxO1*$^{+/+}$ and *FoxO1*$^{fl/fl}$ mice. Data are pooled from three independent experiments.

(C) Luciferase reporter assays for *Pdcd1* promoter activity in Jurkat T cells after overnight stimulation with PMA plus ionomycin transfected with MigR1 empty vector (EV) or a constitutively active FoxO1 (FoxO1^{AAA}). Data are pooled from two independent experiments.

(D) Representative histograms and cumulative bar graphs showing PD-1, Eomes, and T-bet expression in P14+ CTLs retrovirally transduced with EV (gray) or FoxO1^{AAA} (GFP+) (red) and examined at day 15 after LCMV-Cl13 infection. Data are pooled from three independent experiments (n = 6–9; each group).

(E) Representative dot plots of P14+ CTLs retrovirally transduced with EV (gray) or FoxO1^{AAA} (red) showing PD-1 versus Eomes (top) or PD-1 versus T-bet (bottom) expression as in (D).

Error bars are mean ± SEM.

we identified FoxO1 as a critical rheostat downstream of mTORC2 that controls the expression of PD-1 and Bcl-2 and the balance between Eomes^{hi} and T-bet^{hi} CTL populations to maintain the overall homeostasis of virus-specific CTLs during chronic viral infection. These data support the notion that suppression of AKT and mTOR and augmentation of PD-1 expression via increased FoxO1 is both a normal and necessary part of the progression of CTL exhaustion that serves not only to prevent excessive immunopathology, but also to sustain virus-specific CTLs during persistent antigenic stimulation.

Our study, along with that of Sullivan et al. (2012b), also brings to light the nonredundant and apparent contrasting roles of FoxO1 and FoxO3a in regulating CTL responses during chronic infection. Although the phospho-flow analysis was unable to distinguish between phosphorylated FoxO1 and FoxO3a, other data using FoxO1-specific antibodies clearly demonstrated the enhanced expression and nuclear retention of FoxO1 protein in PD-1^{hi} CTLs; the specific phosphorylation state and nuclear localization of FoxO3a remains to be investigated. Additionally, whereas FoxO1 was necessary to maintain Bcl-2 expression and CTL survival, FoxO3a promoted Bim expression and CTL apoptosis during chronic LCMV infection (Grayson et al., 2006; Sullivan et al., 2012b). Furthermore, FoxO3a does not appear to share the same role as FoxO1 in promoting the differentiation of more terminally exhausted PD-1^{hi}Eomes^{hi} CTLs (Sullivan et al., 2012b). These findings suggest that FoxO1 and FoxO3a can regulate both overlapping and divergent aspects of CD8+ T cell biology, an idea that is further supported by pathway analysis of predicted FoxO1 and FoxO3a target genes in CTLs after acute and chronic viral infection (data not shown). Additionally, this work highlights important dichotomies in the transcriptional targets of FoxO1 in CD8+ T cells between acute and chronic infection. For example, FoxO1 controls expression of *Il7r*, *Ccr7*, *Sell* (CD62L), and *Tcf7* in long-lived memory CTLs (Kim et al., 2013; Hess Michelini et al., 2013; Rao et al., 2012; Tejera et al., 2013) that are downregulated in exhausted CTLs during chronic infection. Similar observations have been made with regard to the transcriptional targets of

T-bet and Eomes in CTLs from acute versus chronic LCMV infection (Doering et al., 2012). These data underscore the potential role for distinct epigenetic changes and/or unique transcriptional networks in regulating how these and other transcription factors might have altered functions in the context of a resolving versus a persistent infection. In the future, it will be important to determine the transcriptional program and target genes regulated by FoxO1 and FoxO3a and how and why these might vary in the different settings of acute and chronic infection.

Importantly, this study outlines a positive-feedback pathway regulating the differentiation and homeostasis of exhausted CTLs during chronic infection, wherein decaying TCR responsiveness, mediated in part by PD-1, lowered PI3K, AKT, and mTOR signaling. HIF-1 is an important downstream target of mTORC1 and thus our work provides greater understanding of why constitutive HIF-1 activity would sustain aerobic glycolysis and effector functions in CTLs during chronic LCMV infection (Doedens et al., 2013). Further, our work identifies that decreased mTORC2 activity consequently led to elevated FoxO1 activity, which repressed GzmB but sustained PD-1, Eomes, and Bcl2 expression. Thus, FoxO1 aids in the formation and maintenance of more terminally exhausted $CD8^+$ T cells. Identification of FoxO1 as a transcriptional activator of *Pdcd1* (PD-1) is an important finding because little is known about the regulation of this locus. Although our data show that FoxO1 can directly bind to a known regulatory element of *Pdcd1* and that $FoxO1^{AAA}$ can promote the differentiation of $PD\text{-}1^{hi}$ CTLs during chronic LCMV infection, it is likely that the drop in PD-1 expression in $FoxO1^{fl/fl}$ CTLs depends in part on increased T-bet expression because T-bet can repress *Pdcd1* transcription (Kao et al., 2011; Lu et al., 2014; Hess Michelini et al., 2013; Paley et al., 2012). Although FoxO1 has not yet been found to bind to the *Tbx21* (T-bet) locus directly (Rao et al., 2012), T-bet expression and activity are, nevertheless, markedly increased in the absence of FoxO1 (Hess Michelini et al., 2013; Rao et al., 2012; Tejera et al., 2013). During chronic infection, it is plausible that FoxO1 and T-bet inhibit each other's binding in the C region of the *Pdcd1* locus, thereby imposing divergent effects on PD-1 expression and CTL exhaustion. Alternatively, given the density of NFAT binding sites in the *Pdcd1* locus, FoxO1 might cooperate with NFAT to regulate and/or sustain the expression of PD-1 in the presence of persistent antigen during chronic infection (Lu et al., 2014; Oestreich et al., 2008). This idea is similar to the role proposed for NFAT:FoxO1 complexes that coordinate the expression and activity of FoxP3 during $CD4^+$ regulatory T cell differentiation (Samstein et al., 2012). We favor a model in which NFAT activity initiates PD-1 expression, whereas FoxO1 might help to sustain its expression directly and indirectly through its ability to regulate or compete with T-bet. Together, these data outline a unique transcription factor network that might regulate the expression of PD-1 and the differentiation of exhausted CTLs (Doering et al., 2012).

The FoxO family of transcription factors have a plethora of binding partners and can undergo numerous posttranslational modifications (Calnan and Brunet, 2008). In response to nutrient deprivation, phosphorylation of FoxO transcription factors by AMPK on residues distinct from that of AKT redirects its transcriptional activity to different targets (Greer and Brunet, 2005; Greer et al., 2007). Additionally, acetylation of FoxO1 by CBP and its deacetylation by Sirt1 can also regulate its activity and specificity, the latter being suggestive of FoxO1's role in regulating mitochondrial function and lipid oxidation (Gross et al., 2008). Indeed, future proteomic studies of FoxO1 might help to decipher how its activity can be differentially regulated between acute and chronic infection and the types of cofactors it interacts with in T cells. Additionally, our study has provided proof of concept that manipulation of FoxO1, and possibly other members of the PI3K, AKT, and mTOR pathway, can regulate the expression and function of PD-1 in exhausted CTLs that could lead to therapeutic options for fighting chronic viral infection or cancer. However, our data would argue that fine-tuning of FoxO1 activities and/or temporal regulation thereof, as opposed to their complete blockade, might serve as more rational therapeutic design.

EXPERIMENTAL PROCEDURES

Mice and LCMV Infections

C57BL/6j mice from NCI and Thy-1.1^+ P14 TCR transgenic mice (Pircher et al., 1989) that recognize the $H\text{-}2D^b$ gp33 epitope were used where indicated. $FoxO1^{fl/fl}$ mice (Ouyang et al., 2009) were bred in house onto *Gzmb-cre* or P14 *Gzmb-cre* mice (Cui et al., 2011). Mice were intraperitoneally (i.p.) infected with 2×10^5 plaque forming units (p.f.u.) LCMV Armstrong (Arm)—which causes an acute infection—or intravenously (i.v.) with 2×10^6 p.f.u. LCMV Clone 13 (Cl13)—which causes a chronic infection. Viral titers were determined by plaque assays as previously described (Cui et al., 2011). The use of all animals was conducted in accordance with Yale University IACUC guidelines.

Flow Cytometry, Amnis, and Phospho-flow

Cells were surface or intracellular stained with commercially available antibodies and kits from eBioscience or BD Biosciences. Importantly, for all signaling experiments, cells were rested for a period of 2–4 hr at 37°C in 1% RPMI medium to allow for any signals to return to background prior to peptide, α-CD3, or cytokine restimulation. Intracellular FoxO1 and phosphorylated (p) $p\text{-}AKT^{308}$, $p\text{-}AKT^{473}$, $p\text{-}S6^{235/236}$, and $p\text{-}FoxO1/3a^{24/32}$, $p\text{-}Zap70^{318}/p\text{-}Syk^{352}$, and $p\text{-}ERK^{202/204}$ were detected via paraformaldehyde fixation and methanol permeabilization and antibodies from Cell Signaling. Primary unconjugated antibodies used in phospho-flow and total FoxO1 staining were detected by secondary staining with anti-rabbit IgG 647 antibody (Molecular Probes). Nuclear FoxO1 localization was performed with Amnis Imagestream and analyzed with Imagestream software. 2-NBDG, mitochondrial green, and mitochondrial deep-red staining (Invitrogen) was performed by incubating splenocytes in normal or glucose-free (for 2-nbdg staining) RPMI media at 37°C for 10 min, washed, stained for surface markers, and immediately analyzed via flow cytometry.

Chromatin Immunoprecipitation

In brief, $\sim 10 \times 10^6$ cells were crosslinked with 1% formaldehyde and quenched with 0.125 M glycine as previously described (Ouyang et al., 2012). Nuclei were isolated with 0.1% Triton buffer and sonicated in SDS lysis buffer to ~300–500 bp size. A total of 5 μg of antibody was complexed overnight to anti-rabbit magnetic beads (Invitrogen), and 100 μg of chromatin was used per immunoprecipitation (IP) reaction. FoxO1 ChIP antibody (ab39670) was obtained from Abcam. Control rabbit-IgG was obtained from Santa Cruz Biotechnology. Samples were washed with low-salt, high-salt, LiCl, and TE buffers, eluted with SDS and reverse crosslinked overnight, followed by proteinase K digestion and DNA purification. Samples were then subjected to quantitative PCR with published primer sets (Oestreich et al., 2008). A region outside of the promoter region (X-region) was used as a negative control (forward, 5′-CAGTATGCAGCTCCTGTCTCC-3′; reverse, 5′-ACACCATGACCAAACCCAAG-3′), and FoxO1 KO P14 CTLs were used to control for antibody-IP specificity. Fold enrichment was calculated over rabbit IgG control.

Retroviral Transduction

P14 TCR transgenic mice were superinfected i.v. with 2 × 10^6 p.f.u. of LCMV-Arm. After 24 hr, P14 T cells were spin-transduced with MigR1-EV GFP or MigR1-FoxO1^{AAA} GFP retrovirus (a kind gift from T. Unterman, University of Illinois at Chicago) and a small number (1–5 × 10^3) of cells were adoptively transferred into LCMV-Cl13-infected recipients (Hand et al., 2010; Kao et al., 2011).

Luciferase Reporter Assays

In brief, Jurkat T cells were cotransfected with firefly luciferase plasmids containing either the *Pdcd1* promoter B-region or B+C region (a kind gift from J. Boss, Emory University) and a control Renilla luciferase plasmid via Fugene 6 transfection reagent (Promega). Cells were either left unstimulated or stimulated for 18 hr with PMA plus ionomycin to activate PD-1 luciferase reporter activity as previously reported (Oestreich et al., 2008). Luciferase activity was determined by DualGlo Luciferase reagent (Promega). "Fold over control" *Pdcd1* promoter luciferase activity was determined by normalizing to Renilla luciferase activity of stimulated over unstimulated samples.

Seahorse Extracelluar Flux Analysis

Seahorse analysis experiments were performed as previously described (Henao-Mejia et al., 2013). In brief, day 8 P14 CTLs (Thy1.1$^+$) were purified from LCMV-Arm- or LCMV-Cl13-infected mice by positive selection with magnetic bead (Stem Cell Technologies) to greater than 90% purity, and plated on a pretreated poly-D-lysine-coated 96-well polystyrene Seahorse plate. Cells were allowed to equilibrate at 37°C for 30 min prior to starting the assay. Oligomycin (ATPase inhibitor, 0.5 μM) and FCCP (0.2 μM) were injected where indicated and ECAR (mpH/min) oxygen consumption rate (pMoles/min) was measured.

Statistical Analysis

Data were analyzed by the Student's unpaired t test or with one-way ANOVA analysis with Tukey post-test for multiple comparisons with Prism 6. Error bars are the mean ± SEM. An asterisk indicating a p value of less than 0.05 (*$p < 0.05$) is considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.013.

AUTHOR CONTRIBUTIONS

S.M.K. and M.M.S. designed all the experiments, analyzed the data, and wrote the manuscript. S.M.G. assisted with many of the experiments. H.D.M., I.A.P., J.H.C., and C.J.P. provided critical reagents for the experiments. G.C. helped with the design of ChIP experiments. M.O.L. provided the FoxO1-floxed mouse strain, expertise with the ChIP experiments, and editing of the manuscript.

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REFERENCES

Angelosanto, J.M., Blackburn, S.D., Crawford, A., and Wherry, E.J. (2012). Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. J. Virol. *86*, 8161–8170.

Araki, K., Turner, A.P., Shaffer, V.O., Gangappa, S., Keller, S.A., Bachmann, M.F., Larsen, C.P., and Ahmed, R. (2009). mTOR regulates memory CD8 T-cell differentiation. Nature *460*, 108–112.

Baitsch, L., Baumgaertner, P., Devêvre, E., Raghav, S.K., Legat, A., Barba, L., Wieckowski, S., Bouzourene, H., Deplancke, B., Romero, P., et al. (2011). Exhaustion of tumor-specific CD8$^+$ T cells in metastases from melanoma patients. J. Clin. Invest. *121*, 2350–2360.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature *439*, 682–687.

Biggs, W.H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W.K., and Arden, K.C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc. Natl. Acad. Sci. USA *96*, 7421–7426.

Brooks, D.G., McGavern, D.B., and Oldstone, M.B. (2006). Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. J. Clin. Invest. *116*, 1675–1685.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Calnan, D.R., and Brunet, A. (2008). The FoxO code. Oncogene *27*, 2276–2288.

Chang, C.H., Curtis, J.D., Maggi, L.B., Jr., Faubert, B., Villarino, A.V., O'Sullivan, D., Huang, S.C., van der Windt, G.J., Blagih, J., Qiu, J., et al. (2013). Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell *153*, 1239–1251.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., and Riley, J.L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J. Immunol. *173*, 945–954.

Cui, W., Liu, Y., Weinstein, J.S., Craft, J., and Kaech, S.M. (2011). An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity *35*, 792–805.

Doedens, A.L., Phan, A.T., Stradner, M.H., Fujimoto, J.K., Nguyen, J.V., Yang, E., Johnson, R.S., and Goldrath, A.W. (2013). Hypoxia-inducible factors enhance the effector responses of CD8(+) T cells to persistent antigen. Nat. Immunol. *14*, 1173–1182.

Doering, T.A., Crawford, A., Angelosanto, J.M., Paley, M.A., Ziegler, C.G., and Wherry, E.J. (2012). Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity *37*, 1130–1144.

Fife, B.T., Pauken, K.E., Eagar, T.N., Obu, T., Wu, J., Tang, Q., Azuma, M., Krummel, M.F., and Bluestone, J.A. (2009). Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat. Immunol. *10*, 1185–1192.

Finlay, D., and Cantrell, D.A. (2011). Metabolism, migration and memory in cytotoxic T cells. Nat. Rev. Immunol. *11*, 109–117.

Finlay, D.K., Sinclair, L.V., Feijoo, C., Waugh, C.M., Hagenbeek, T.J., Spits, H., and Cantrell, D.A. (2009). Phosphoinositide-dependent kinase 1 controls migration and malignant transformation but not cell growth and proliferation in PTEN-null lymphocytes. J. Exp. Med. *206*, 2441–2454.

Finlay, D.K., Rosenzweig, E., Sinclair, L.V., Feijoo-Carnero, C., Hukelmann, J.L., Rolf, J., Panteleyev, A.A., Okkenhaug, K., and Cantrell, D.A. (2012). PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. J. Exp. Med. *209*, 2441–2453.

Francisco, L.M., Salinas, V.H., Brown, K.E., Vanguri, V.K., Freeman, G.J., Kuchroo, V.K., and Sharpe, A.H. (2009). PD-L1 regulates the development,

maintenance, and function of induced regulatory T cells. J. Exp. Med. *206*, 3015–3029.

Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H., and Thompson, C.B. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity *16*, 769–777.

Frebel, H., Nindl, V., Schuepbach, R.A., Braunschweiler, T., Richter, K., Vogel, J., Wagner, C.A., Loffing-Cueni, D., Kurrer, M., Ludewig, B., and Oxenius, A. (2012). Programmed death 1 protects from fatal circulatory failure during systemic virus infection of mice. J. Exp. Med. *209*, 2485–2499.

Grayson, J.M., Weant, A.E., Holbrook, B.C., and Hildeman, D. (2006). Role of Bim in regulating CD8+ T-cell responses during chronic viral infection. J. Virol. *80*, 8627–8638.

Greer, E.L., and Brunet, A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene *24*, 7410–7425.

Greer, E.L., Oskoui, P.R., Banko, M.R., Maniar, J.M., Gygi, M.P., Gygi, S.P., and Brunet, A. (2007). The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J. Biol. Chem. *282*, 30107–30119.

Gross, D.N., van den Heuvel, A.P., and Birnbaum, M.J. (2008). The role of FoxO in the regulation of metabolism. Oncogene *27*, 2320–2336.

Hand, T.W., Cui, W., Jung, Y.W., Sefik, E., Joshi, N.S., Chandele, A., Liu, Y., and Kaech, S.M. (2010). Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. Proc. Natl. Acad. Sci. USA *107*, 16601–16606.

Hedrick, S.M., Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Stone, E.L. (2012). FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. *12*, 649–661.

Henao-Mejia, J., Williams, A., Goff, L.A., Staron, M., Licona-Limón, P., Kaech, S.M., Nakayama, M., Rinn, J.L., and Flavell, R.A. (2013). The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. Immunity *38*, 984–997.

Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Hedrick, S.M. (2013). Differentiation of CD8 memory T cells depends on Foxo1. J. Exp. Med. *210*, 1189–1200.

Jacobs, S.R., Herman, C.E., Maciver, N.J., Wofford, J.A., Wieman, H.L., Hammen, J.J., and Rathmell, J.C. (2008). Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. J. Immunol. *180*, 4476–4486.

Kao, C., Oestreich, K.J., Paley, M.A., Crawford, A., Angelosanto, J.M., Ali, M.A., Intlekofer, A.M., Boss, J.M., Reiner, S.L., Weinmann, A.S., and Wherry, E.J. (2011). Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. Nat. Immunol. *12*, 663–671.

Keir, M.E., Butte, M.J., Freeman, G.J., and Sharpe, A.H. (2008). PD-1 and its ligands in tolerance and immunity. Annu. Rev. Immunol. *26*, 677–704.

Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat. Immunol. *10*, 176–184.

Kerdiles, Y.M., Stone, E.L., Beisner, D.R., McGargill, M.A., Ch'en, I.L., Stockmann, C., Katayama, C.D., and Hedrick, S.M. (2010). Foxo transcription factors control regulatory T cell development and function. Immunity *33*, 890–904.

Kim, E.H., Sullivan, J.A., Plisch, E.H., Tejera, M.M., Jatzek, A., Choi, K.Y., and Suresh, M. (2012). Signal integration by Akt regulates CD8 T cell effector and memory differentiation. J. Immunol. *188*, 4305–4314.

Kim, M.V., Ouyang, W., Liao, W., Zhang, M.Q., and Li, M.O. (2013). The transcription factor Foxo1 controls central-memory CD8+ T cell responses to infection. Immunity *39*, 286–297.

Lu, P., Youngblood, B.A., Austin, J.W., Mohammed, A.U., Butler, R., Ahmed, R., and Boss, J.M. (2014). Blimp-1 represses CD8 T cell expression of PD-1 using a feed-forward transcriptional circuit during acute viral infection. J. Exp. Med. *211*, 515–527.

Macintyre, A.N., Finlay, D., Preston, G., Sinclair, L.V., Waugh, C.M., Tamas, P., Feijoo, C., Okkenhaug, K., and Cantrell, D.A. (2011). Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism. Immunity *34*, 224–236.

Michalek, R.D., and Rathmell, J.C. (2010). The metabolic life and times of a T-cell. Immunol. Rev. *236*, 190–202.

Mueller, S.N., Vanguri, V.K., Ha, S.J., West, E.E., Keir, M.E., Glickman, J.N., Sharpe, A.H., and Ahmed, R. (2010). PD-L1 has distinct functions in hematopoietic and nonhematopoietic cells in regulating T cell responses during chronic infection in mice. J. Clin. Invest. *120*, 2508–2515.

Oestreich, K.J., Yoon, H., Ahmed, R., and Boss, J.M. (2008). NFATc1 regulates PD-1 expression upon T cell activation. J. Immunol. *181*, 4832–4839.

Ouyang, W., Beckett, O., Flavell, R.A., and Li, M.O. (2009). An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. Immunity *30*, 358–371.

Ouyang, W., Beckett, O., Ma, Q., Paik, J.H., DePinho, R.A., and Li, M.O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat. Immunol. *11*, 618–627.

Ouyang, W., Liao, W., Luo, C.T., Yin, N., Huse, M., Kim, M.V., Peng, M., Chan, P., Ma, Q., Mo, Y., et al. (2012). Novel Foxo1-dependent transcriptional programs control T(reg) cell function. Nature *491*, 554–559.

Paley, M.A., Kroy, D.C., Odorizzi, P.M., Johnnidis, J.B., Dolfi, D.V., Barnett, B.E., Bikoff, E.K., Robertson, E.J., Lauer, G.M., Reiner, S.L., and Wherry, E.J. (2012). Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science *338*, 1220–1225.

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S.V., Linsley, P.S., Thompson, C.B., and Riley, J.L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol. Cell. Biol. *25*, 9543–9553.

Patsoukis, N., Li, L., Sari, D., Petkova, V., and Boussiotis, V.A. (2013). PD-1 increases PTEN phosphatase activity while decreasing PTEN protein stability by inhibiting casein kinase 2. Mol. Cell. Biol. *33*, 3091–3098.

Pearce, E.L., and Pearce, E.J. (2013). Metabolic pathways in immune cell activation and quiescence. Immunity *38*, 633–643.

Pearce, E.L., Walsh, M.C., Cejas, P.J., Harms, G.M., Shen, H., Wang, L.S., Jones, R.G., and Choi, Y. (2009). Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature *460*, 103–107.

Pircher, H., Bürki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature *342*, 559–561.

Powell, J.D., and Delgoffe, G.M. (2010). The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity *33*, 301–311.

Powell, J.D., Pollizzi, K.N., Heikamp, E.B., and Horton, M.R. (2012). Regulation of immune responses by mTOR. Annu. Rev. Immunol. *30*, 39–68.

Puglielli, M.T., Zajac, A.J., van der Most, R.G., Dzuris, J.L., Sette, A., Altman, J.D., and Ahmed, R. (2001). In vivo selection of a lymphocytic choriomeningitis virus variant that affects recognition of the GP33-43 epitope by H-2Db but not H-2Kb. J. Virol. *75*, 5099–5107.

Rao, R.R., Li, Q., Odunsi, K., and Shrikant, P.A. (2010). The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. Immunity *32*, 67–78.

Rao, R.R., Li, Q., Gubbels Bupp, M.R., and Shrikant, P.A. (2012). Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. Immunity *36*, 374–387.

Riley, J.L. (2009). PD-1 signaling in primary T cells. Immunol. Rev. *229*, 114–125.

Samstein, R.M., Arvey, A., Josefowicz, S.Z., Peng, X., Reynolds, A., Sandstrom, R., Neph, S., Sabo, P., Kim, J.M., Liao, W., et al. (2012). Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. Cell *151*, 153–166.

Sheppard, K.A., Fitz, L.J., Lee, J.M., Benander, C., George, J.A., Wooters, J., Qiu, Y., Jussif, J.M., Carter, L.L., Wood, C.R., and Chaudhary, D. (2004). PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett. *574*, 37–41.

Shin, H., Blackburn, S.D., Blattman, J.N., and Wherry, E.J. (2007). Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J. Exp. Med. *204*, 941–949.

Shin, H., Blackburn, S.D., Intlekofer, A.M., Kao, C., Angelosanto, J.M., Reiner, S.L., and Wherry, E.J. (2009). A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. Immunity *31*, 309–320.

Speiser, D.E., Utzschneider, D.T., Oberle, S.G., Münz, C., Romero, P., and Zehn, D. (2014). T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion? Nat. Rev. Immunol. *14*, 768–774.

Sullivan, J.A., Kim, E.H., Plisch, E.H., Peng, S.L., and Suresh, M. (2012a). FOXO3 regulates CD8 T cell memory by T cell-intrinsic mechanisms. PLoS Pathog. *8*, e1002533.

Sullivan, J.A., Kim, E.H., Plisch, E.H., and Suresh, M. (2012b). FOXO3 regulates the CD8 T cell response to a chronic viral infection. J. Virol. *86*, 9025–9034.

Tejera, M.M., Kim, E.H., Sullivan, J.A., Plisch, E.H., and Suresh, M. (2013). FoxO1 controls effector-to-memory transition and maintenance of functional CD8 T cell memory. J. Immunol. *191*, 187–199.

Tomasoni, R., Basso, V., Pilipow, K., Sitia, G., Saccani, S., Agresti, A., Mietton, F., Natoli, G., Colombetti, S., and Mondino, A. (2011). Rapamycin-sensitive signals control TCR/CD28-driven Ifng, Il4 and Foxp3 transcription and promoter region methylation. Eur. J. Immunol. *41*, 2086–2096.

van der Windt, G.J., O'Sullivan, D., Everts, B., Huang, S.C., Buck, M.D., Curtis, J.D., Chang, C.H., Smith, A.M., Ai, T., Faubert, B., et al. (2013). CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. Proc. Natl. Acad. Sci. USA *110*, 14336–14341.

Virgin, H.W., Wherry, E.J., and Ahmed, R. (2009). Redefining chronic viral infection. Cell *138*, 30–50.

Wang, R., Dillon, C.P., Shi, L.Z., Milasta, S., Carter, R., Finkelstein, D., McCormick, L.L., Fitzgerald, P., Chi, H., Munger, J., and Green, D.R. (2011). The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity *35*, 871–882.

Wei, F., Zhong, S., Ma, Z., Kong, H., Medvec, A., Ahmed, R., Freeman, G.J., Krogsgaard, M., and Riley, J.L. (2013). Strength of PD-1 signaling differentially affects T-cell effector functions. Proc. Natl. Acad. Sci. USA *110*, E2480–E2489.

Wherry, E.J. (2011). T cell exhaustion. Nat. Immunol. *12*, 492–499.

Yokosuka, T., Takamatsu, M., Kobayashi-Imanishi, W., Hashimoto-Tane, A., Azuma, M., and Saito, T. (2012). Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J. Exp. Med. *209*, 1201–1217.

Zinselmeyer, B.H., Heydari, S., Sacristán, C., Nayak, D., Cammer, M., Herz, J., Cheng, X., Davis, S.J., Dustin, M.L., and McGavern, D.B. (2013). PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. J. Exp. Med. *210*, 757–774.